Chapter 1

Human Chromosomes

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History

- The age of human cytogenetics did not begin until the 1950s, when Tjio and Levan and Ford and Hamerton established the diploid human chromosome number of 46. Lejeune et al. discovered trisomy 21 in Down syndrome, while Ford et al. and Jacobs and Strong established that Turner and Klinefelter syndromes were caused by X-chromosomal anomalies.

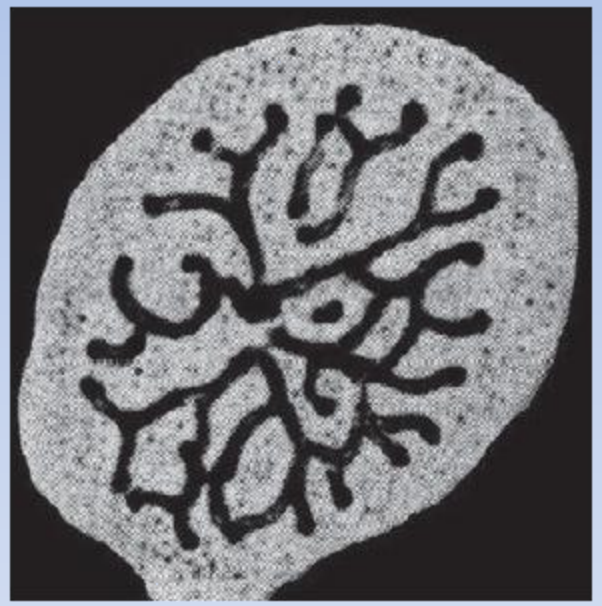


Fig. 3.1 One of the first images of human chromosomes made by the German pathologist J. Arnold in 1879. Arnold examined carcinoma and sarcoma cells because their voluminous nucleus facilitated analysis. The drawing shows a human sarcoma cell.

- ***Birth of Human Cytogenetics 1963–1956***
- ***Introduction of Banding Technologies from the Late 1960s to the Present***
- ***The Birth of Molecular Cytogenetics in the Late 1960s***
- ***Molecular Cytogenetics or Fluorescence In Situ Hybridization 1980 to Date***
- ***Array Technologies: New Dimensions in Resolution from 1997 to Date***

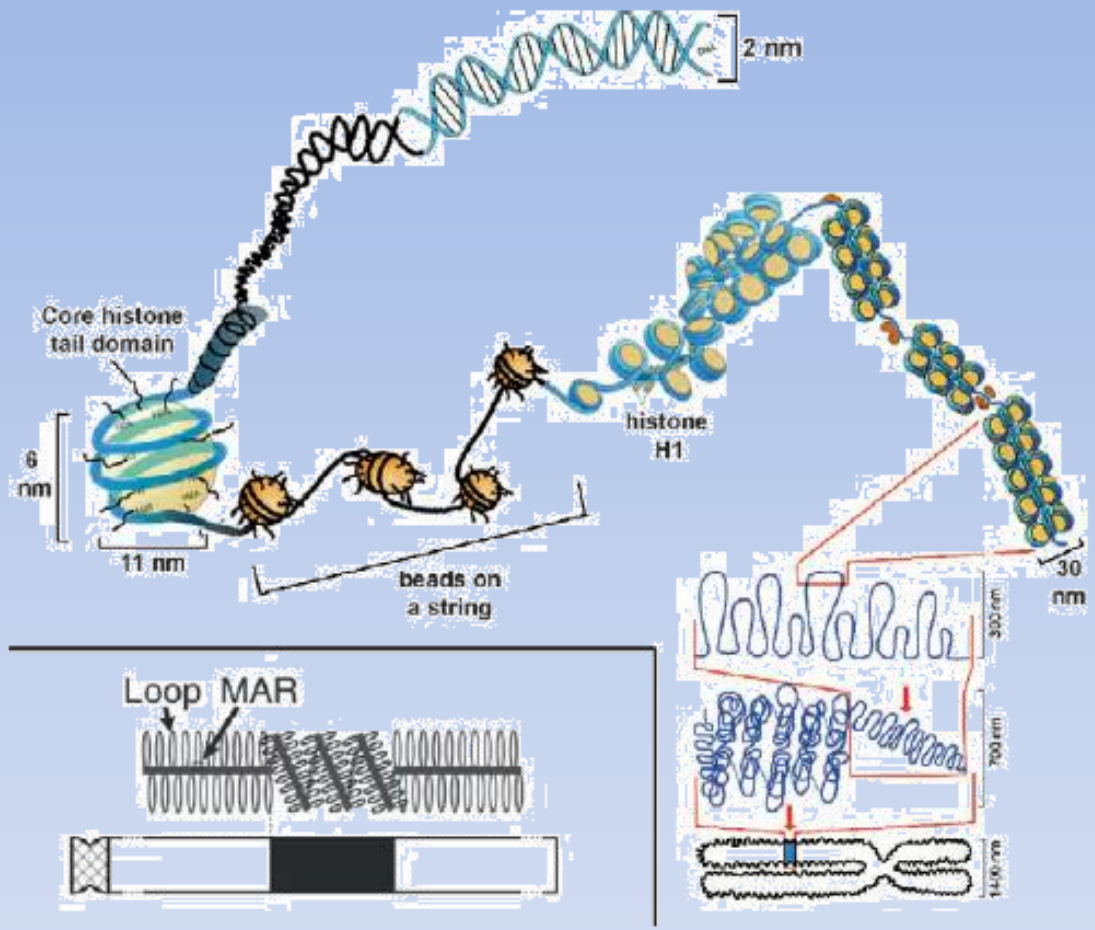


Fig. 3.4 Folding and packing of chromosomal DNA. Scheme of different levels of packing of DNA from the double helix to a metaphase chromosome. (Modified from [109]). *Inset:* Model of a metaphase chromatin structure. Adapted from [202]

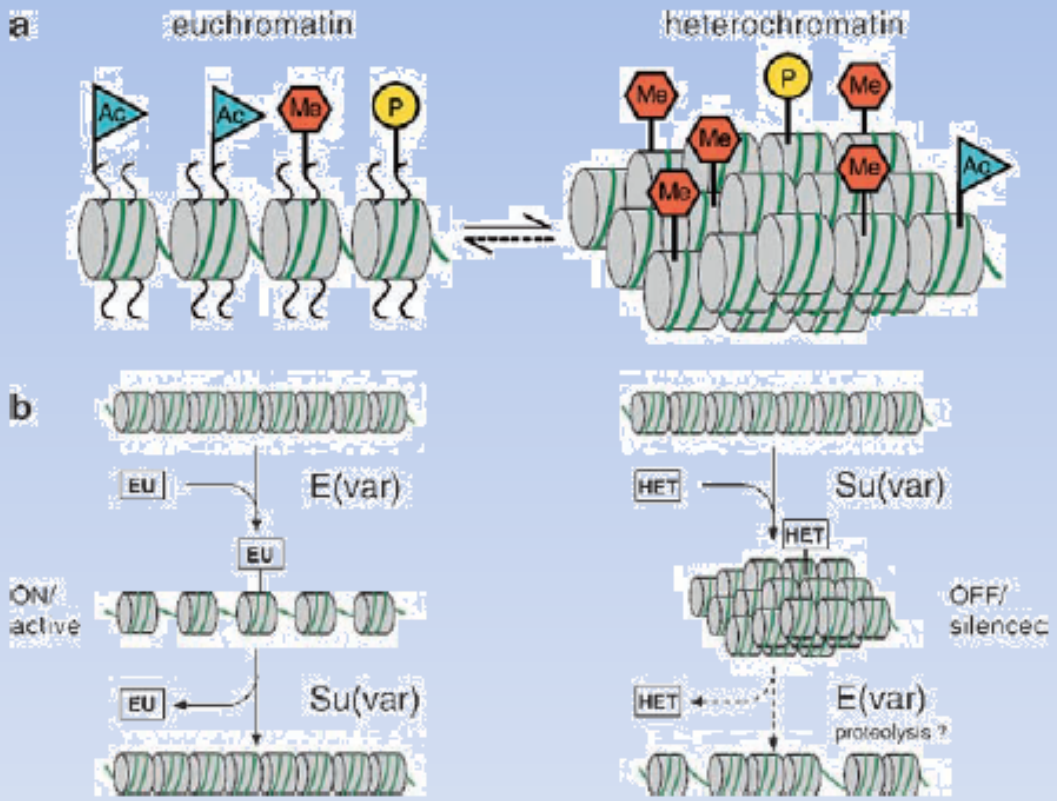
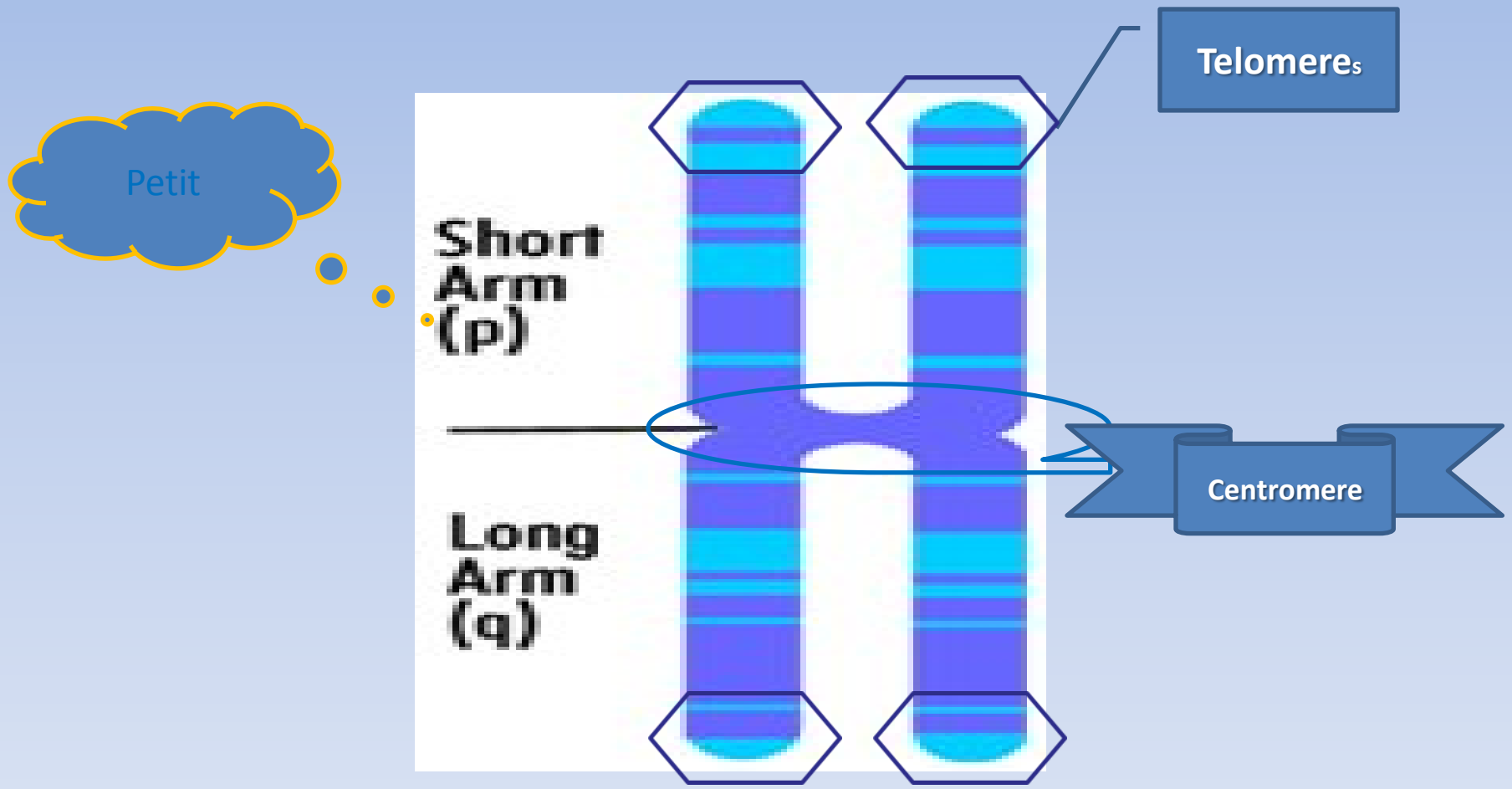


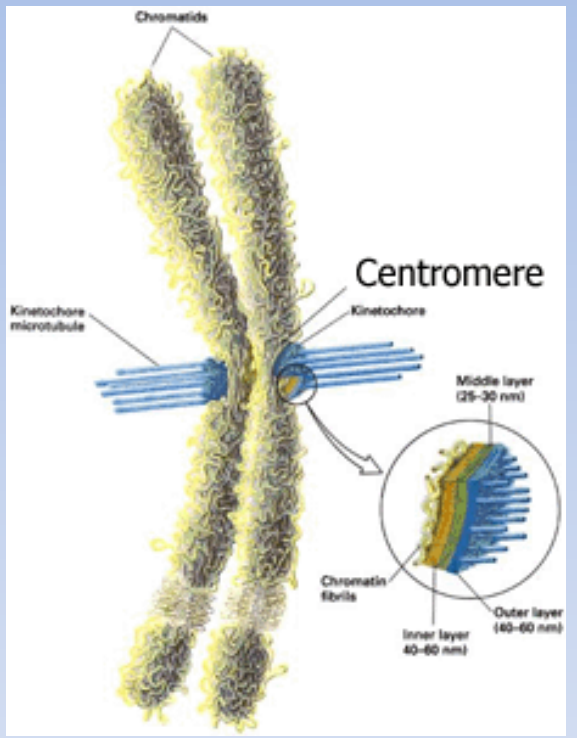
Fig. 3.6 (a, b) Models for euchromatin and heterochromatin histone tail modifications. (From [121]) (a) Schematic representation of euchromatin and heterochromatin as accessible or condensed nucleosome fibers containing acetylated (*Ac*), phosphorylated (*P*), and methylated (*Me*) histone NH_2 termini. (b) Model for adding euchromatic (*EU*) or heterochromatic (*HET*) modification marks onto a nucleosomal template. The position of a gene in an accessible (euchromatic) or an inaccessible (heterochromatic) chromatin environment has also been referred to as position-effect variegation (*PEV*). PEV modifiers can enhance variegation [*E(var)*] or suppress variegation [*Su(var)*]. The *left-hand side* depicts an example in which a region is made accessible, while the *right-hand side* shows the reverse, i.e., transfer into inaccessible heterochromatin. Both processes are reversible

Human Chromosome

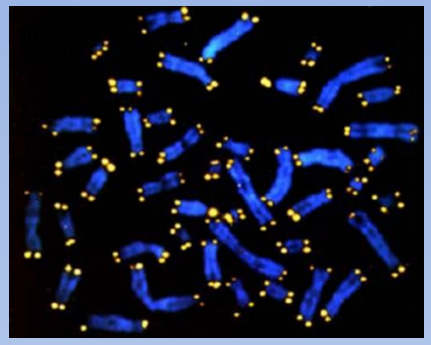


Centromere

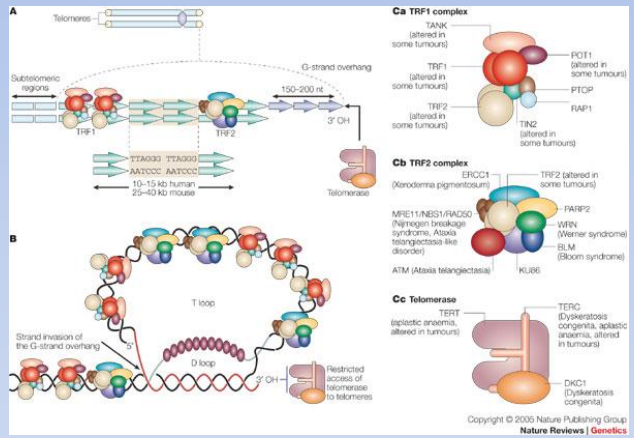
The centromere is the part of a chromosome that links sister chromatids. During mitosis, spindle fibers attach to the centromere via the kinetochore. Centromeres were first defined as genetic loci that direct the behavior of chromosomes. Their physical role is to act as the site of assembly of the kinetochore - a highly complex multiprotein structure that is responsible for the actual events of chromosome segregation - e.g. binding microtubules and signaling to the cell cycle machinery when all chromosomes have adopted correct attachments to the spindle, so that it is safe for cell division to proceed to completion (i.e. for cells to enter anaphase).

- 

Telomere



- The telomere repeats consist of 250–1,500 G-rich tandem sequences (5 ‘-TTAGGG-3‘), which are highly conserved among different species. Telomeric DNA of mammalian cells is formed from the repeating hexanucleotide sequence 5’ -TTAGGG-3’ in one strand (the “G-rich” strand) and the complementary 5 ‘-CCCTAA-3’ in the other (the “C-rich” strand). The G-rich strand is longer by one hundred to several hundred nucleotides, resulting in a long 3’ single strand overhang , thus creating a “sticky end.”
- The end of the single-stranded overhanging region has to be put away in order to avoid initiation of the cellular repair machinery. The 3’ overhanging end of the G-rich strand is annealed to a small region of the C-rich strand, causing the formation of a displacement loop, while the telomeric duplex DNA forms a loop (T-loop). The T-loop together with the displacement loop helps to protect the ends of linear DNA molecules.



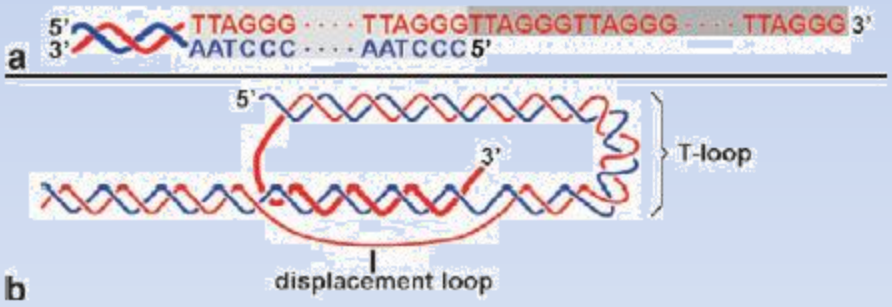


Fig. 3.12 (**a**) The G-rich strand of telomeric DNA (*red*) extends beyond the C-rich strand (*blue*). This creates a 3′ overhang (*dark gray shaded area*). This single stranded 3′ overhang may have a length of several hundred nucleotides. The double-stranded portion of telomeric DNA (*light gray shaded area*) has a length of 5–10 kb. (**b**) The single-stranded 3′ overhang is annealed to a small region of the C-rich strand, causing the formation of a displacement loop while the telomeric duplex DNA forms the T-loop. Adapted from [250]

Function of telomeres

- Protects chromosomes from recombination
- Protects chromosomes from end-to-end fusion
- Protects chromosomes from recognition as damaged DNA
- Provides a means for complete replication of chromosomes
- Contributes to the functional organization of chromosomes within the nucleus
- Participates in the regulation of gene expression
- Serves as a molecular clock that controls the replicative capacity of human cells and their entry into senescence

Telomerase

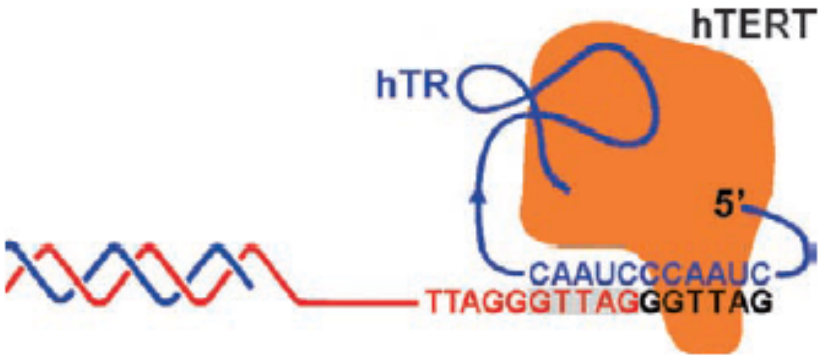
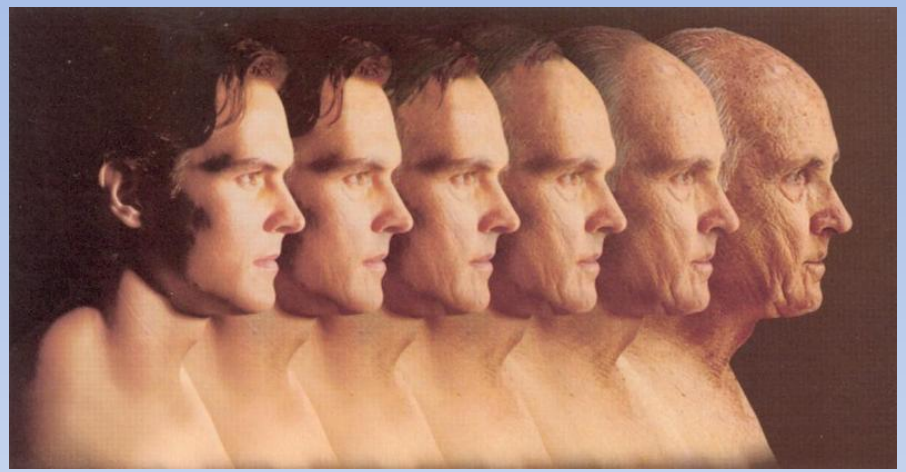


Fig. 3.14 Illustration of the two essential subunits of the human telomerase holoenzyme: the hTERT catalytic subunit (*orange*) and the associated hTR subunit (*blue*), which can elongate telomeres. The 3′ end of chromosomes (*red letters*) is extended by reverse-transcribing the template region of the telomerase RNA. Near the RNA 5′ end are sequences complementary to telomeric DNA repeat sequences (*blue letters*). A short nucleotide sequence of this RNA pairs with terminal DNA sequences (*gray shaded red letters*). The adjacent RNA nucleotides provide the template for adding nucleotides to the 3′ end of the chromosome (*black letters*). Repetition of this process in an iterative fashion makes it possible for telomeres to be elongated. Adapted from [250]

Alternative lengthening of telomeres (ALT)

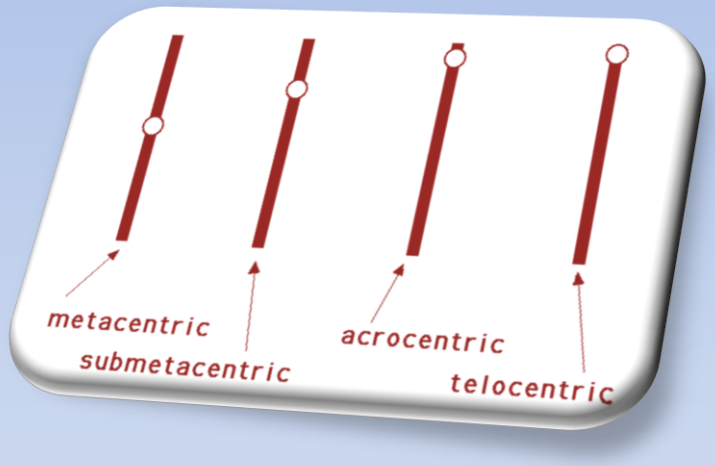
- In the absence of telomerase, telomeric DNA loss can also be compensated for by alternative lengthening of telomeres (ALT) mechanisms. The ALT mechanisms are still unclear and appear to rely on homologous recombination, rolling-circle replication, extrachromosomal circle integration, and break-induced replication.
- ALT-positive cells have a number of characteristics dissimilar from those in cells that use telomerase for telomere maintenance. Telomeres in ALT-positive cells are typically quite long and heterogeneously sized compared with the shorter, more homogeneous population of telomeres usually present in telomerase-positive cells. Based on differences in telomere structure, mechanisms that amplify subtelomeric repeats and mechanisms that lengthen the simple telomeric repeats alone can be distinguished. The ALT pathways can be considered as backups of telomere maintenance in organisms that normally exploit the telomerase system.
- For instance, whereas ALT is inhibited in normal human cells, some human tumors maintain their telomeres using ALT.

Telomere shortening and aging



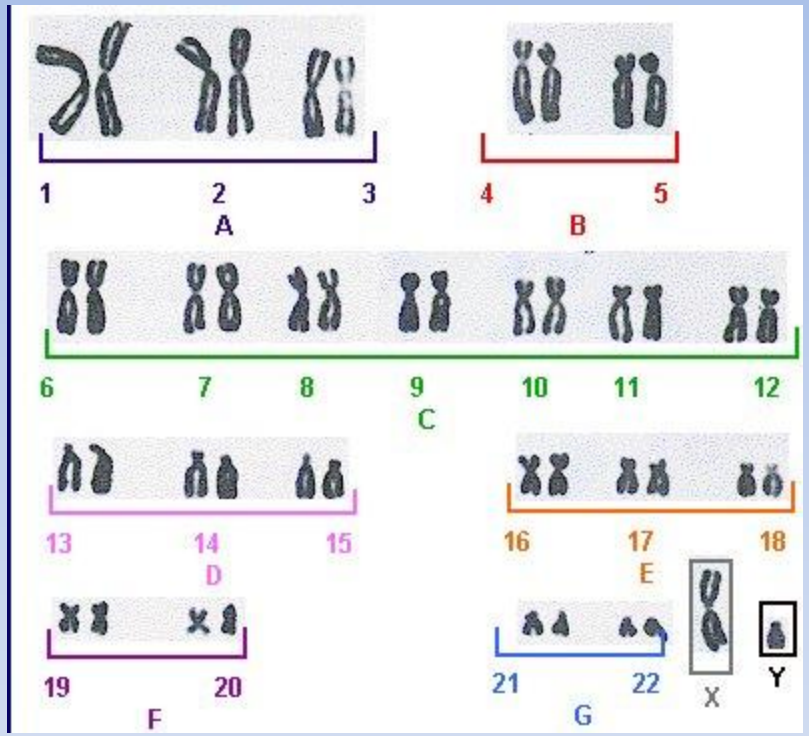
Chromosome types

- Metacentric p=q
- Submetacentric p<q
- Acrocentric p<<q
- Telocentric p=0

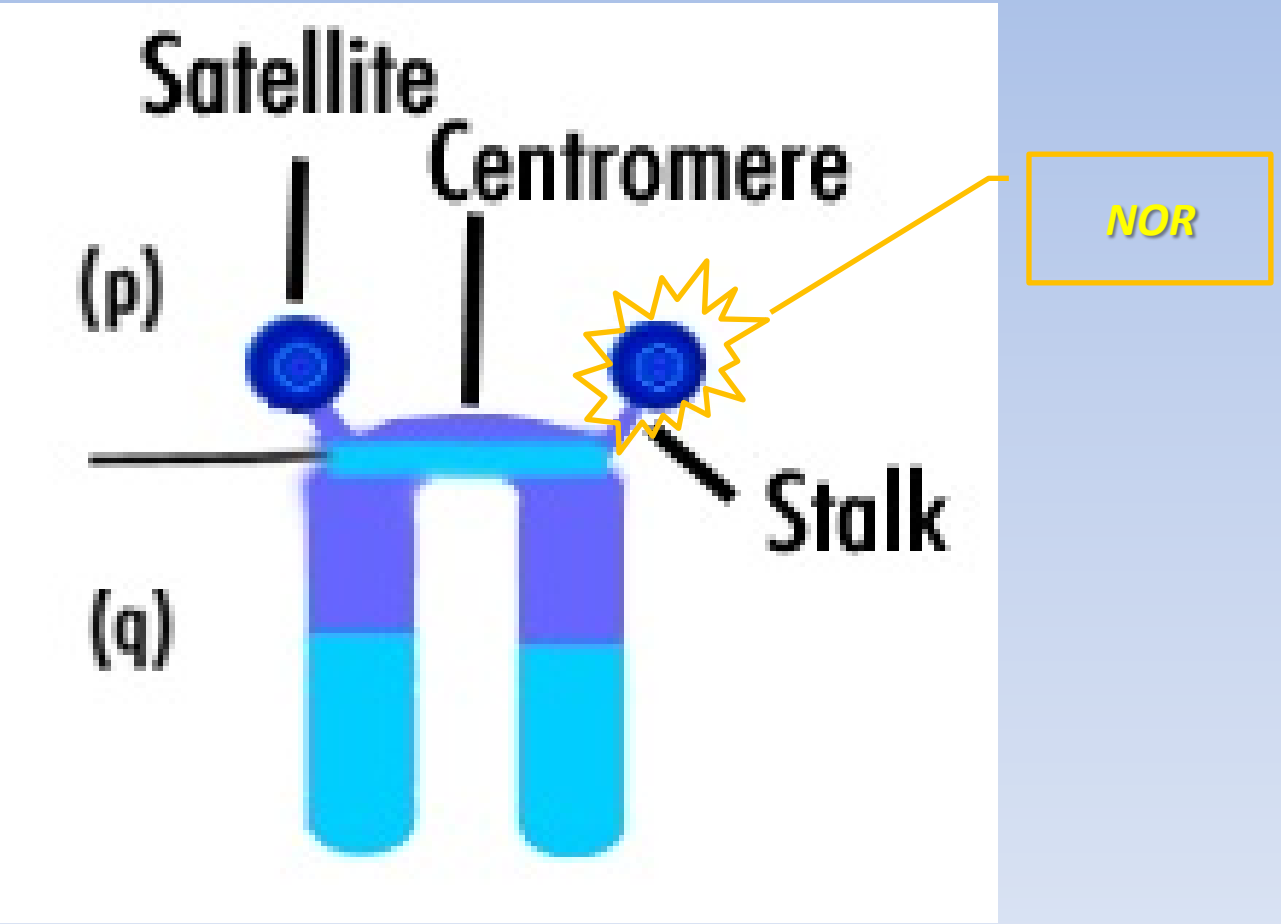


Human Chromosome groups

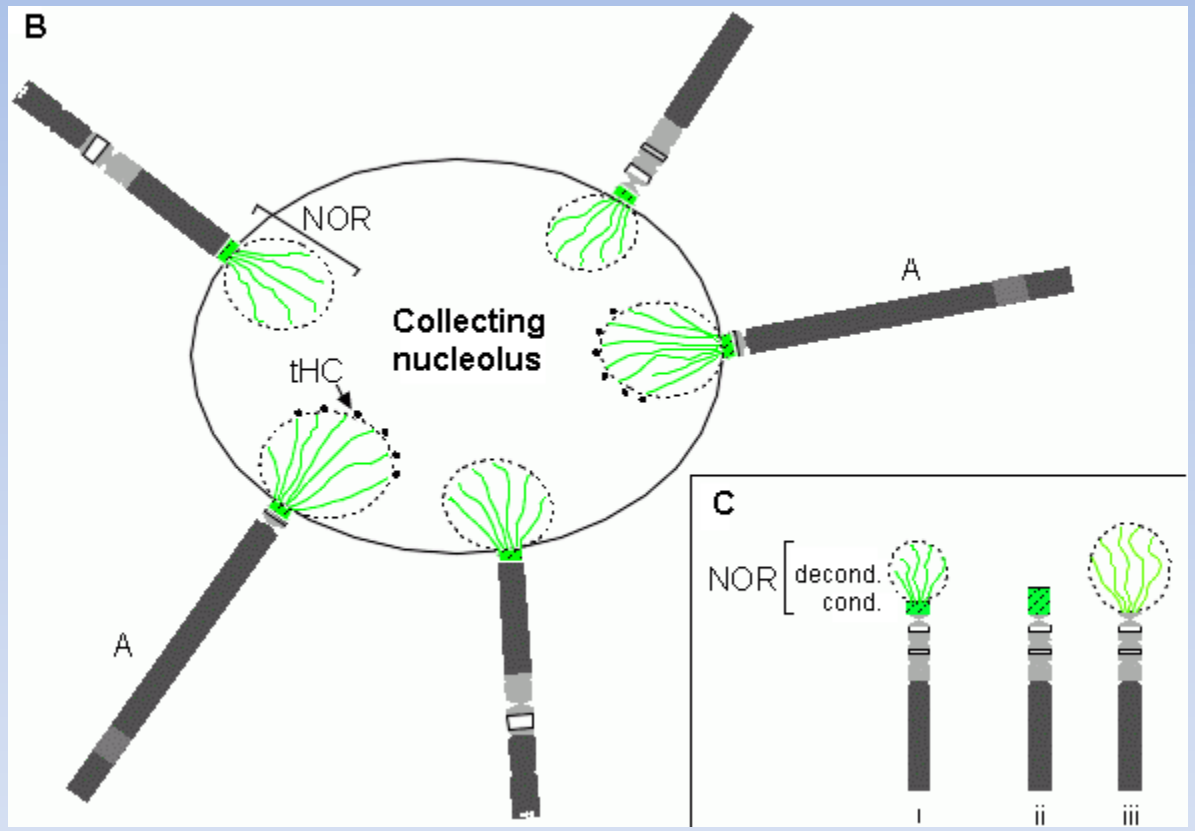
- A: 1-3, MC (2=SMC)
- B: 4,5, SMC
- C: 6-12,X , SMC
- D: 13-15 , AC
- E: 16-18 , SMC
- F: 19,20 , MC
- G: 21,22,Y , AC



Acrocentric chromosomes



Nucleolus



NOR: Nucleolus Organizing Region

Chromosome Analysis Methods

G-banding

G-banding is a technique used in cytogenetics to produce a visible karyotype by staining condensed chromosomes. It is useful for identifying genetic diseases through the photographic representation of the entire chromosome complement. The metaphase chromosomes are treated with trypsin (to partially digest the chromosome) and stained with Giemsa. Dark bands that take up the stain are strongly A,T rich (gene poor). The reverse of G-bands is obtained in R-banding. Banding can be used to identify chromosomal abnormalities, such as translocations, because there is a unique pattern of light and dark bands for each chromosome.

It is difficult to identify and group chromosomes based on simple staining because the uniform color of the structures makes it difficult to differentiate between the different chromosomes. Therefore, techniques like G-banding were developed that made "bands" appear on the chromosomes. These bands were the same in appearance on the homologous chromosomes, thus, identification became easier and more accurate. The acid-saline-Giemsa protocol reveals G-bands.

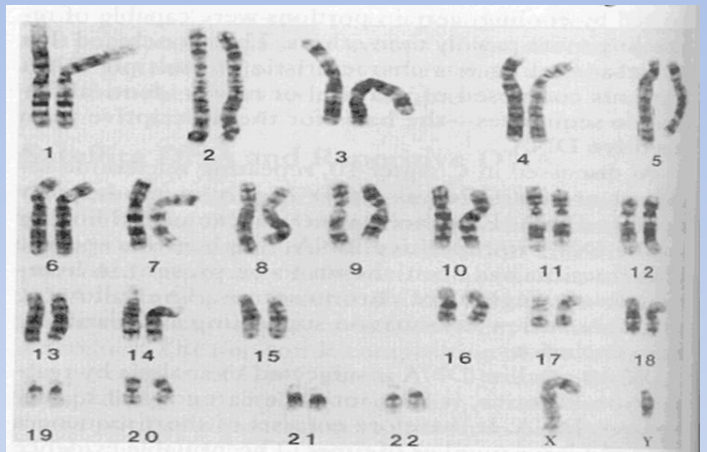
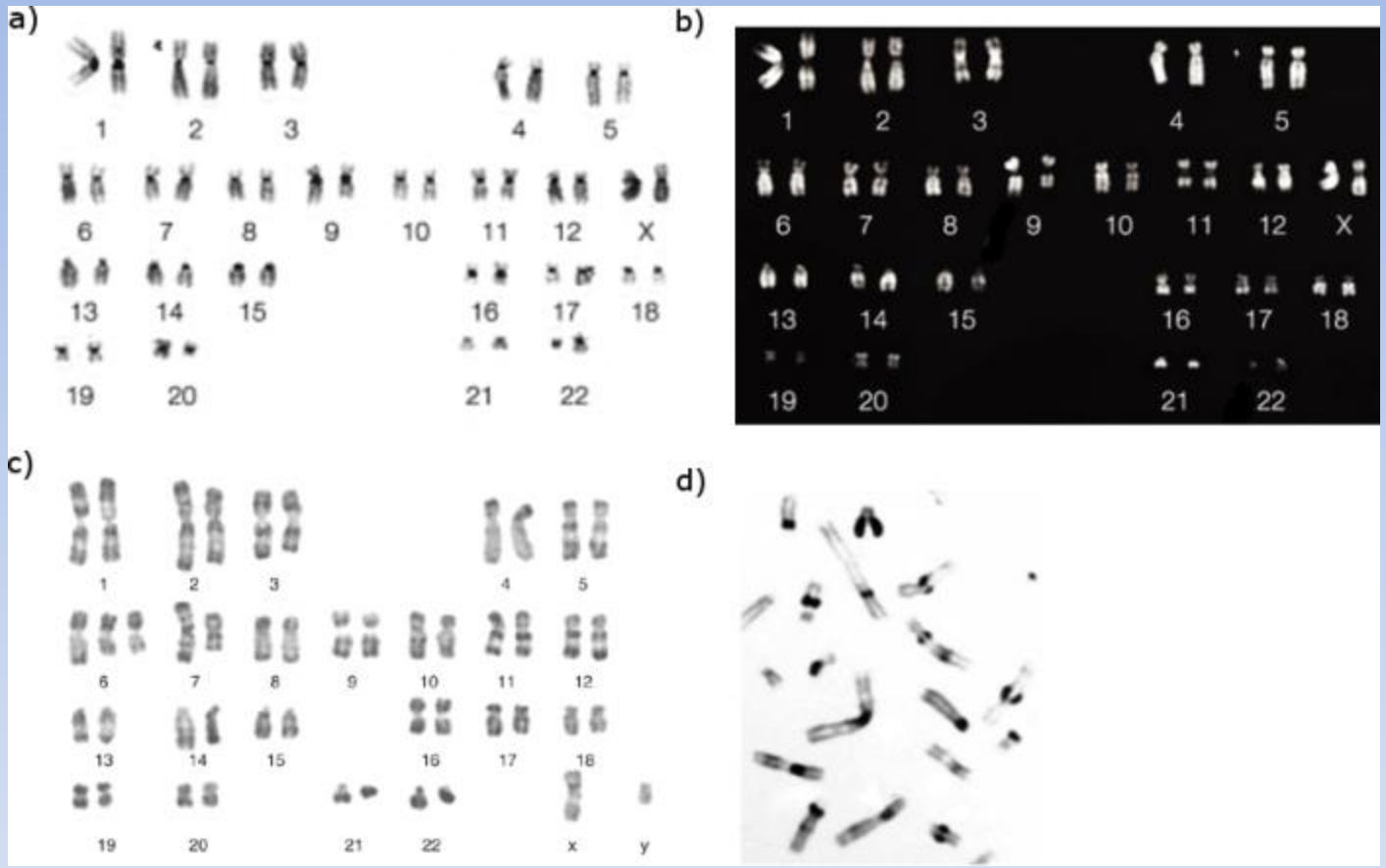


FIGURE 17.13 G-banded karyotype of a normal human male showing approximately 400 bands per haploid set of chromosomes. Chromosomes were derived from cells in metaphase.

R-, Q-, C-banding



Different chromosomal staining techniques reveal variations in chromosome structure. Cytogeneticists use these patterns to recognize the differences between chromosomes and enable them to link different disease phenotypes to chromosomal abnormalities. Giemsa banding (a), Q-banding (b), R-banding (c) and C-banding (d)

In Silico -*Generated Bands*

- More recently, new approaches have been used to achieve *in silico chromosome staining. Using the DNA* sequences of the draft human genome and sophisticated computer software which assigns gray values to chromosomal regions depending on the percentage of the GC content, it has proved possible to achieve successful reconstruction of bands resembling Giemsa bands . Such *in silico approaches will probably* improve with further refinements of draft human genome sequences and should result in the most reliable schematic representations of chromosomes.
- *In silico chromosome staining provided the solution* to an old problem. As already mentioned, Giemsa-dark and -light bands are generally thought to correspond to GC-poor and GC-rich regions, respectively; however, several experiments have shown that the correspondence is quite poor. *In silico banding clearly shows that* Giemsa-dark bands are *locally GC-poor regions compared* with the flanking regions, but not compared with the entire genome. These findings are consistent with the model that MARs, which are known to be AT rich, are present more densely in Giemsa-dark bands than in Giemsa-light bands .
- In fact, G-bands are the regions in which the GC content is only lower relative to the surrounding regions, and not relative to the entire genome

Fig. 3.11 Giemsa and *in silico* bands for all chromosomes. Short (p) and long (q) arms are positioned *left* and *right*, respectively. Giemsa bands obtained from Francke [81] are shown in *black pictograms*. The bands depicted in *black*, *gray*, and *white* represent euchromatins, and the darkness of each band reflects the shading. Pericentromeric heterochromatin and heteromorphic regions of chromosomes 1, 3, 9, 16, 19, and Y are depicted by *crosshatched* and *horizontal lines*, respectively. *In silico* bands constructed by using windows of 2.5 and 9.3 Mb are shown in *blue*. The *thin lines* between Giemsa and *in silico* bands denote aligned G-bands. From [170], copyright 2002, *National Academy of Sciences*, USA

Ag-NOR Bands

- Ag-NOR banding aims at the specifi c staining of the chromosomal regions that form and maintain the nucleoli in interphase nuclei, the so-called nucleolus organizing regions (NORs) . These regions consist of multiple copies of DNA sequences or genes for ribosomal RNA and are located at the short arms of acrocentric chromosomes. The “Ag” indicates that the staining is done by silver impregnation. Ag-NOR staining refl ects the transcriptional activity of the NORs. Thus, frequently not all p-arms of the acrocentric chromosomes show staining. Most individuals have four to seven active NORs per cell.

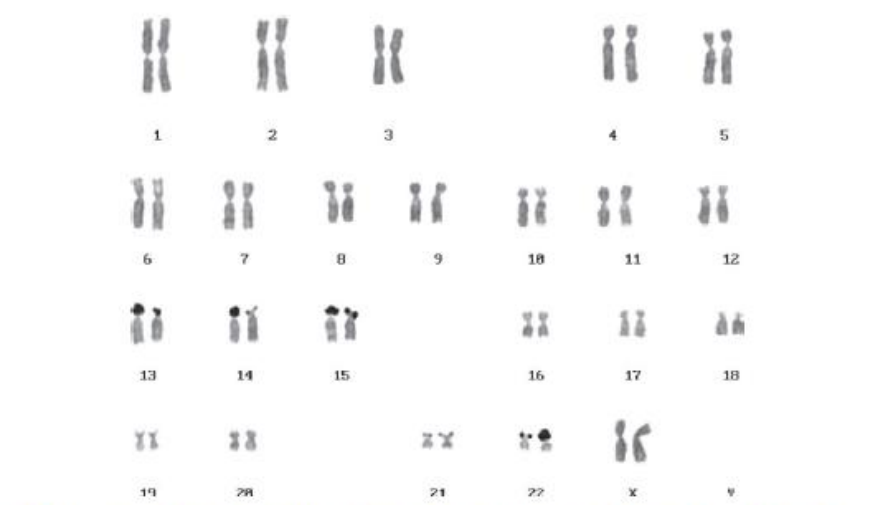


Fig. 3.23 AgNOR-banding of a normal female metaphase. Staining is usually visible at most but not at all p-arms of the acrocentric chromosomes

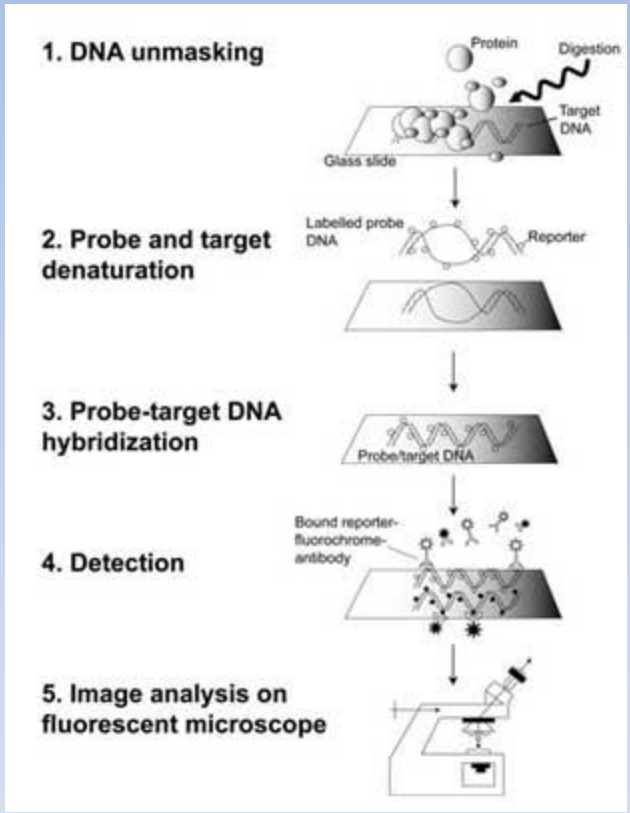
DA/DAPI Staining

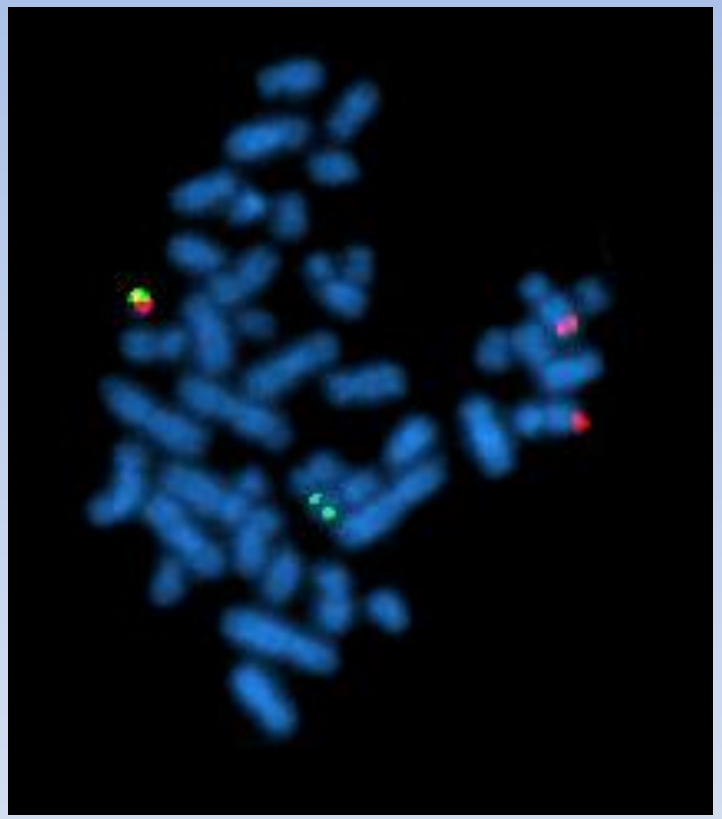
- DA/DAPI staining involves exposure to the nonfluorescent counterstain distamycin A (DA), followed by staining with fluorescent 4’-6-diamidino-2-phenylindole (DAPI).
- As a result, certain heterochromatic regions will appear brightly fluorescent.

ISH (In Situ Hybridization)

- In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA or RNA strand (i.e., probe) to localize a specific DNA or RNA sequence in a portion or section of tissue (in situ), or, if the tissue is small enough (e.g. plant seeds, Drosophila embryos), in the entire tissue (whole mount ISH), in cells and in circulating tumor cells (CTCs). This is distinct from immunohistochemistry, which usually localizes proteins in tissue sections. DNA ISH can be used to determine the structure of chromosomes.

FISH (Fluorescent ISH)





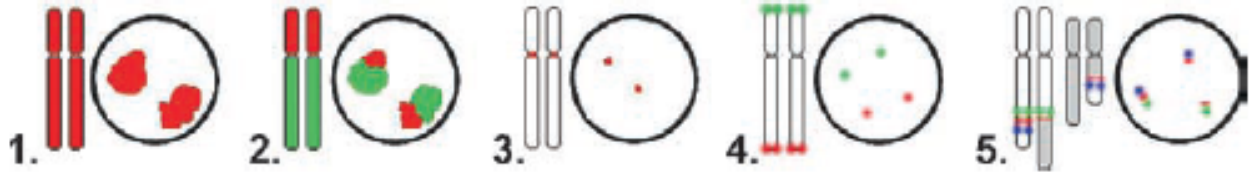
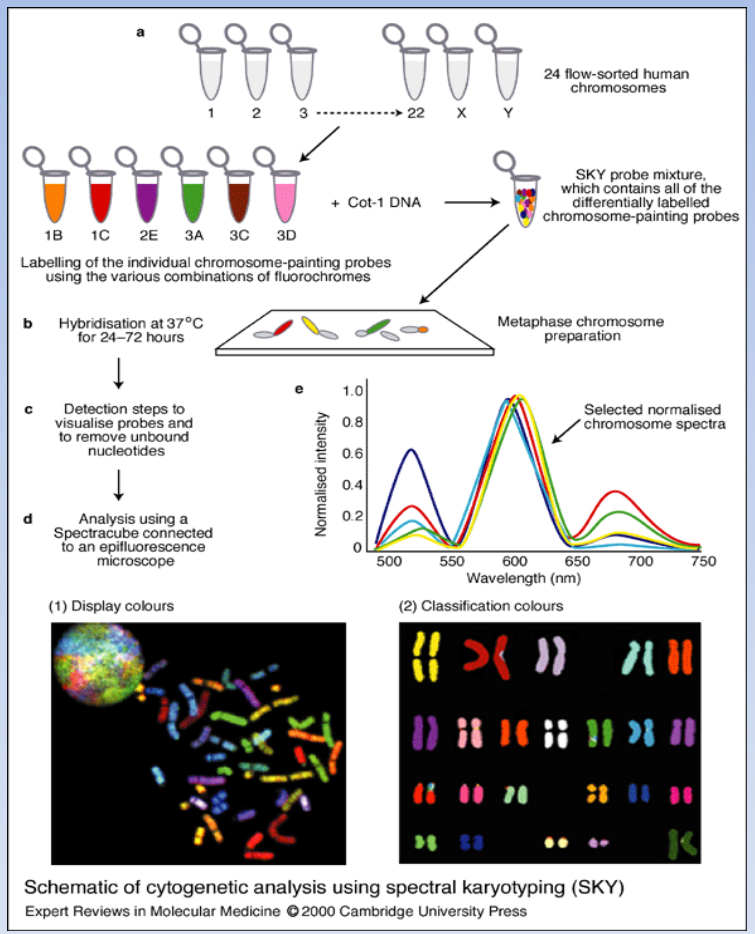


Fig. 3.25 A variety of different DNA probe types are available for hybridization to both metaphase chromosomes and interphase nuclei. The panel shows some examples: *1:* A painting probe stains the entire chromosome. In a normal diploid interphase nucleus two chromosome territories are visible. *2:* Microdissection allows the generation of probes specific for any region within the genome. Differentially labeled chromosome arm-specific probes are shown in this example. In interphase nuclei, the differently labeled chromosome arms within a chromosome territory can be distinguished. *3:* For almost all human chromosomes, probes specific for the centromere are available. Hybridization of these highly repetitive probes does not require suppression with Cot-1 DNA. Owing to their ease of use and high signal intensities, these probes are very popular for the counting of chromosome copy number in interphase nuclei. *4:* For almost any region within the genome, region-specific large insert clones are available. The example shows subtelomeric probes, which are often used to screen for cryptic translocations. Clones for other regions can easily be obtained from publicly available resources. *5:* For known structural rearrangements, special probe sets can be designed to facilitate diagnosis. In this example, the probe set includes a breakpoint-spanning probe and two breakpoint-flanking probes. Use of this probe set allows the structural rearrangement to be detected even in interphase nuclei

SKY (Spectral Karyotyping)

- Spectral karyotyping is a molecular cytogenetic technique used to simultaneously visualize all the pairs of chromosomes in an organism in different colors. Fluorescently labeled probes for each chromosome are made by labeling chromosome-specific DNA with different fluorophores. Because there are a limited number of spectrally-distinct fluorophores, a combinatorial labeling method is used to generate many different colors. Spectral differences generated by combinatorial labeling are captured and analyzed by using an interferometer attached to a fluorescence microscope. Image processing software then assigns a pseudo color to each spectrally different combination, allowing the visualization of the individually colored chromosomes.



Schematic of cytogenetic analysis using spectral karyotyping (SKY)

SKY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COBRA-FISH

- The ability to probe for the location of DNA sequences in morphologically preserved chromosomes and nuclei by fluorescence in situ hybridization (FISH) provided for cytogenetics a quantum leap forward in resolution and ease of detection of chromosomal aberrations. COBRA-FISH, an acronym for COmbined Binary RAtio-FISH is a multicolor FISH methodology, which enables recognition of all human chromosome arms on the basis of color, thus greatly facilitating cytogenetic analysis. It also permits gene and viral integration site mapping in the context of chromosome arm painting. Here we review the principle, practice and applications of COBRA-FISH.

Interphase FISH

- One important feature of FISH-based assays is their ability to yield information about chromosomes or chromosomal subregions in intact interphase nuclei enabling a technology termed “interphase cytogenetics” . Interphase cytogenetics is useful in diagnostic applications where metaphase spreads cannot be obtained, where only small cell numbers are available, or where large cell numbers are to be screened with a particular probe set. Interphase FISH enables rapid screening of large numbers of cells, such as the screening of tumor cell nuclei with centromere-specific probes to establish the presence of chromosomal instability. With careful selection of probes even structural rearrangements, such as translocations and inversions, can be visualized in interphase nuclei.

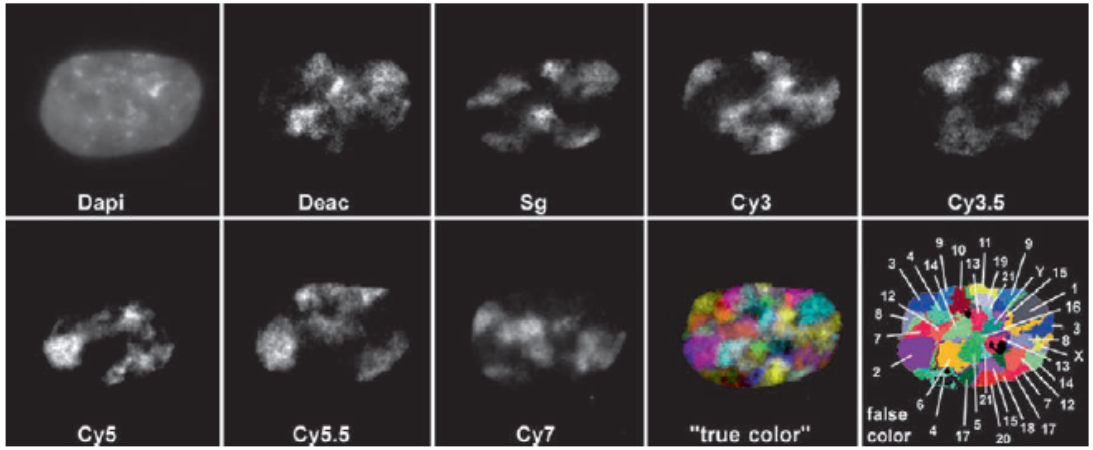

Fig. 3.28 All chromosome domains, each in a different color, can be simultaneously visualized in intact interphase nuclei to study the higher order 3D organization of the genome. The image illustrates a deconvoluted mid-plane nuclear section through a male human G_0 fibroblast nucleus. DAPI is applied to stain the nucleus. For the labeling of chromosome painting probes seven different fluorochromes (diethylaminocoumarin [*DEAC*], spectrum green [*SG*], and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5, and Cy7) were used. Here, each channel represents the painting of a subset of chromosome painting probes with the respective fluorochrome. A RGB image of the 24 differently labeled chromosomes (1-22, X, and Y) was produced by superposition of the seven channels. As in 24-color karyotyping, each chromosome has a combinatorial label of a unique fluorochrome combination. Thus, each chromosome territory is amenable to an automated classification so that appropriate software assigns the corresponding chromosome number to a territory. If a stack of these images is collected through a nucleus, a simultaneous 3D reconstruction of all chromosomes is possible. For further details see [31]

comparative genomic hybridization (CGH)

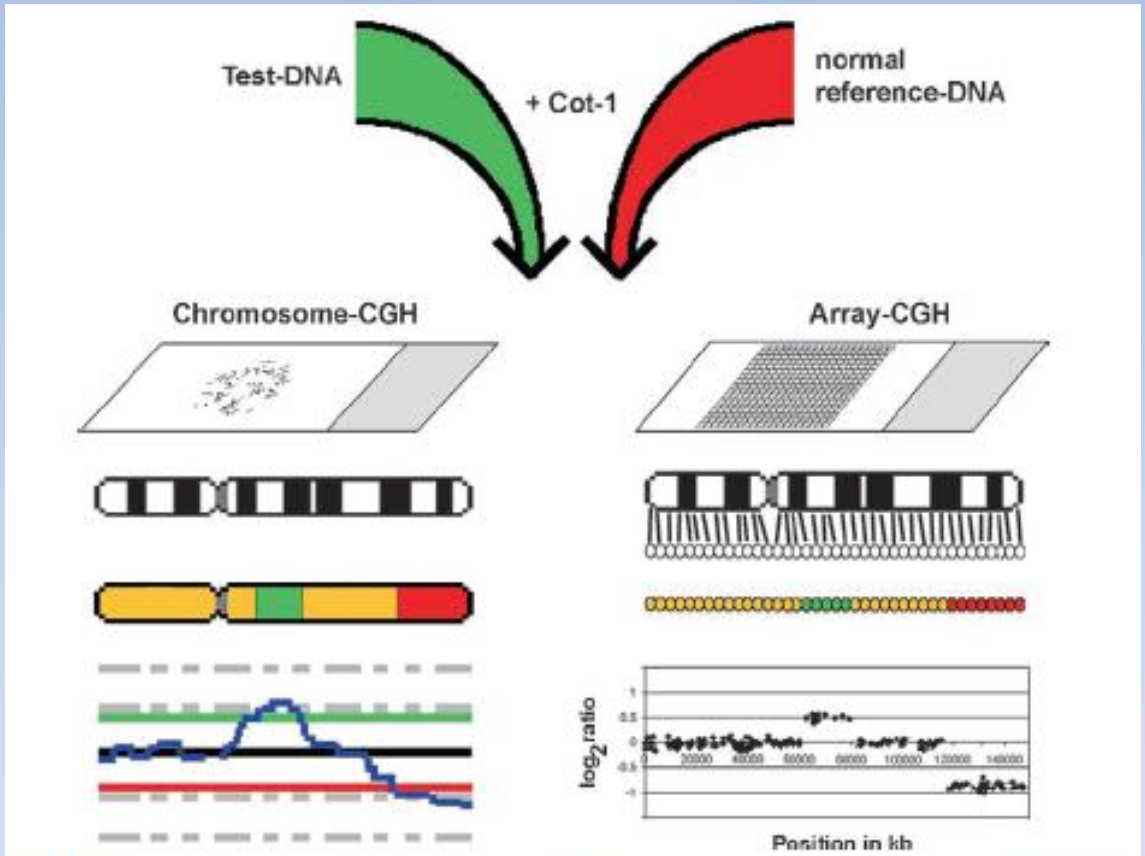

Fig. 3.29 Outline of comparative genomic hybridization (CGH) experiments. CGH maps readily regions of gains and losses in a test-DNA, which is in most cases retrieved from a patient or from a tumor sample. In a CGH analysis, test DNA and normal reference DNA are labeled with different fluorochromes. Here, a test DNA is labeled with a *green* fluorochrome, whereas the reference DNA is labeled with a *red* fluorochrome. In conventional CGH a mixture of these two DNA samples is together with an excess of unlabeled Cot-1 DNA hybridized to normal chromosome spreads (*left-hand side*). In array-CGH/matrix-CGH the hybridization target can consist of large insert clones or oligonucleotides arrayed onto glass slides (*right-hand side*).

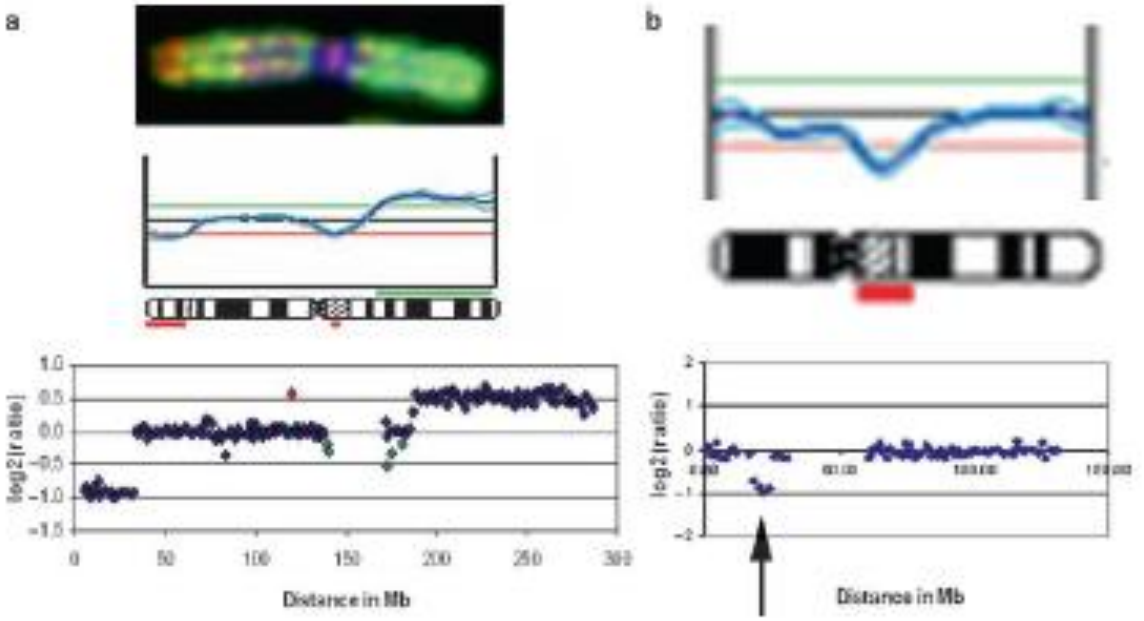

Fig. 3.31 (a, b) Comparison of the different resolution limits of conventional and array-CGH. In this example, DNA from a primary renal cell adenocarcinoma line (769P, ATCC No. CRL-1933) was hybridized to both metaphase spreads and a large-insert clone, i.e., BAC array. (a) The *top panel* illustrates the hybridization pattern on chromosome 1. The distal tip of the p-arm (*left*) appears *red*, which indicates a loss of this region. In contrast, the q-arm (*right*) is extensively stained with *green*, which suggested overrepresentation of this region. The center of chromosome 1 has very low fluorescence signals as this region represents the large chromosome 1 heterochromatin block. As this region consists only of repetitive sequences, hybridization is blocked by the addition of an excess of unlabeled Cot-1 DNA to the hybridization mix. Interpretation of CGH-hybridization patterns does not depend on visual inspection. Instead computer programs are employed, which calculate the intensities of both the *green* and the *red* fluorochrome and the ratio values between these fluorochromes. The result is displayed in a graph, shown in the *center*. The ratio profiles are usually calculated as a mean value of several metaphase spreads. The *three horizontal lines* above the chromosome 1 pictogram represent different values of the fluorescence intensities between the tumor and the reference DNA. The *black line* represents balanced fluorescence intensities, while the *right line* is the threshold for loss and the *green line*, the threshold for a gain of DNA material. The *lower panel* shows the same result obtained on a BAC array. The respective gains and losses are identified with ease. In contrast to conventional CGH, the breakpoints of lost and gained regions can be accurately mapped as they appear as sharp transitions in the ratio profile. (b) Cell line 769P also has a small single-copy deletion on chromosome 9p of about 6.3 Mb. The resolution limits for the detection of deletions or duplications with conventional CGH were estimated to be in the range of about 10 Mb. Consequently, this deletion is not identified with conventional CGH. The "deletion" shown in the *upper panel* is caused by the large heterochromatic block on chromosome 9, which owing to the suppression conditions has no or only very low fluorescence intensities. Such chromosomal regions, rich in repetitive sequences, are prone to resulting in artifacts in chromosome CGH. In array-CGH this heterochromatin block is visible as a large gap around chromosome position 50 Mb because these regions are not represented on arrays. However, the 6.3-Mb deletion is readily visible in array-CGH (*arrow*)

Other approaches for evaluation of copy number changes

- Copy number alterations of specific genomic sequences can also be measured without the need for chromosomes using multiplex amplifiable probe hybridization (MAPH) [and multiplex ligation-dependent probe amplification (MLPA) .
- MAPH is based on the hybridization of specific probes with uniform linkers to denatured genomic DNA immobilized to a nylon membrane. Probes for different regions vary in length and hybridize to the immobilized DNA in proportion to the copy number of the corresponding sequence in the genome. After hybridization and stringent washing the probes bound to the membrane are released and amplified by PCR using a radioactively or fluorescently labeled primer pair that recognizes the linker sequence. After size separation on a gel the relative intensity of the peaks between a test and reference sample is compared to determine the copy number of the target DNA sequence.
- MPLA is similar to MAPH, but hybridization and amplification take place in solution with no need to immobilize the DNA onto a nylon membrane. Each region is represented by two adjacent tailed probes that are joined by a ligation reaction on the target DNA. Subsequent amplification can then only take place from ligated probes as target and not from any other sequence.
- These methods are very rapid and cost-effective and have found favor for specific diagnostic procedures, such as exon deletion screening, but are not easily scaled for whole-genome scanning.

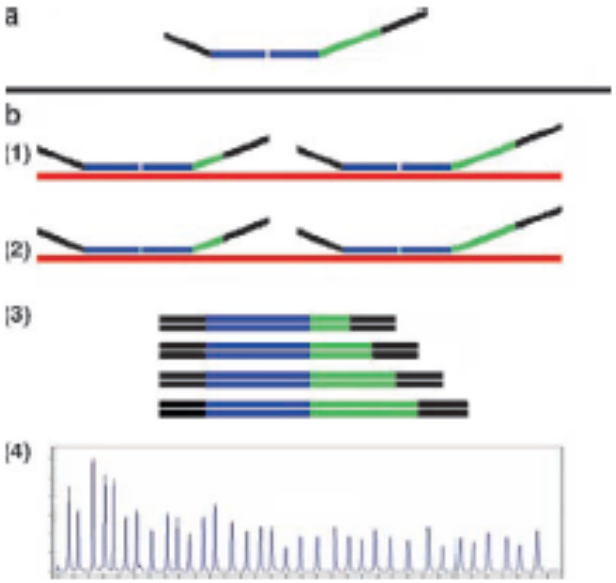

Fig. 3.30 (**a**, **b**) The multiplex ligation-dependent probe amplification (MLPA) procedure. (**a**) Design of a MLPA probe: MLPA probes consist of two separate oligonucleotides, each containing the target sequence (shown in *blue*), a "stuffer sequence" (*green*), and the PCR primer sequences (*black*). The PCR primer sequences are identical for all probes, so that all probes can be later amplified with the same primer set. The stuffer sequence has a different size for each MLPA probe, which allows multiplexing, i.e., multiple probes can be hybridized simultaneously. Different probes are then distinguished by their size. (**b**) The MLPA procedure consists of several steps: *1:* In a first step the DNA to be analyzed is denatured and MLPA probes are hybridized to the DNA. Here, two different MLPA probes are hybridized; the right probe has a larger stuffer sequence than the left probe. *2:* In the second step the ligation reaction takes place. The two probe oligonucleotides hybridize to immediately adjacent target sequences and only then can they be ligated during the ligation reaction. *3:* The ligated probes are amplified by PCR. Probe oligonucleotides that are not ligated only contain one primer sequence and cannot be amplified exponentially. *4:* In the next step amplification products are separated by electrophoresis; and the data can be analyzed. (Adapted from www.mlpa.com)